

AD_____

Award Number: DAMD17-01-1-0190

TITLE: Cellular Imaging Technologies for Discovering Improved
Breast Cancer Therapies

PRINCIPAL INVESTIGATOR: Fred J. Schaufele, Ph.D.

CONTRACTING ORGANIZATION: University of California at San Francisco
San Francisco, California 94143-0962

REPORT DATE: May 2002

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE May 2002	3. REPORT TYPE AND DATES COVERED Annual (16 Apr 01 - 15 Apr 02)
----------------------------------	----------------------------	--

4. TITLE AND SUBTITLE Cellular Imaging Technologies for Discovering Improved Breast Cancer Therapies	5. FUNDING NUMBERS DAMD17-01-1-0190
---	--

6. AUTHOR(S) Fred J. Schaufele, Ph.D.	
--	--

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) University of California at San Francisco San Francisco, California 94143-0962 E-Mail: fred@diabetes.ucsf.edu	8. PERFORMING ORGANIZATION REPORT NUMBER
---	--

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012	10. SPONSORING / MONITORING AGENCY REPORT NUMBER
---	--

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited	12b. DISTRIBUTION CODE
---	------------------------

13. ABSTRACT (Maximum 200 Words) This Idea award was approved to study SERM-selective interactions of estrogen receptors alpha and beta with interacting factors. In this first year, we developed highly integrated cellular imaging techniques that measured not only ligand-regulated co-localization (as proposed) but also, simultaneously, direct interactions via fluorescence resonance energy transfer techniques. With this combined approach, we studied the effects of a spectrum of existing and novel SERMs on the co-localization and interactions of ERalpha with ERbeta and with itself. We found a distinct deficiency in the ability of the soy SERM genistein to promote ERalpha interaction with itself, but not with ERbeta. Other SERMs including raloxifene, ICI182780, tamoxifen, 4-hydroxy-tamoxifen and four chemical derivatives of tamoxifen behaved exactly like estradiol in these assays. A derivative of genistein behaved exactly like genistein. Most importantly, another chemical derivative of genistein behaved like estradiol and the remaining SERMs. This work is now being written for publication. Thus, techniques were developed that distinguished the ligand-specific interactions regulated by existing and novel SERMs in living cells. We look forward to expanding these studies to a broader spectrum of interactions to characterize the precise similarities and differences in the molecular interactions of different SERMs.
--

14. SUBJECT TERMS breast cancer, selective estrogen receptor modulators, fluorescence microscopy	15. NUMBER OF PAGES 21
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Unlimited
---	--	---	---

NSN 7540-01-280-5500

Standard Form 298 (Rev. 2-89)
Prescribed by ANSI Std. Z39-18
298-102

Table of Contents

Cover	1
SF 298	2
Table of Contents	3
Introduction	4
Body	4
Key Research Accomplishments	10
Reportable Outcomes	10
Conclusions	11
References	12
Appendices	13

INTRODUCTION

A subset of breast tumors depends upon circulating estrogens for their growth (1, 2). Drugs, such as tamoxifen, which bind the estrogen receptor (ER) to occlude estrogen binding, have been successful in the treatment of this class of breast tumor (3-5). These drugs have significant side effects owing to their anti-estrogenic effects in other tissues (5-12). In addition, some of these drugs activate, instead of block, ER action in other tissues in which anti-estrogenic behavior is preferred clinically (3, 5, 7, 13).

It is our goal to characterize the molecular, cellular and pharmacologic basis of the tissue-specific actions of these selective estrogen receptor modulators (SERMs). We have developed cellular imaging techniques that precisely characterize the SERM-regulated molecular interactions of the two different isoforms of ER (14, 15). By associating different activities with different functional groups on the pharmacophore, we are identifying the consequences of specific chemical modifications on tissue-selective activities. It is our long-term aim to utilize these precise molecular characterizations to identify and predict SERMs, with unique interaction profiles, that display reduced side effects and enhanced efficacy for breast cancer treatment and preventative therapies.

BODY

Statement of Work

As abridged from our Statement of Work, this project was supported to

“use our innovative imaging techniques to identify similarities and differences in the ability of:

Task 1:representative known ER ligands (DES, coumestrol, ICI 182780, GW7604, GW5638) and
Task 2:novel ER ligands (modifications of tamoxifen, raloxifene, GW5638 and known phytoestrogens)

to promote or block specific alterations in the intranuclear locations of the forty-seven indicated green fluorescent, ER-interacting co-factors, subfragments (peptides) derived from those co-factors and DNA binding sites relative to the intranuclear locations of red fluorescent ER α and ER β .”

General Progress towards Statement of Work

Within the first twelve months of the project, we achieved significant progress towards our goals. We specifically followed the effects of estradiol, tamoxifen, 4-hydroxy tamoxifen, GW 7604, GW5638, ICI 182780, the phytoestrogen genistein and chemical derivatives of tamoxifen and genistein on the co-localization of GFP and RFP-labeled proteins in living cells. Some modifications in the procedures arose from rapid developments in our techniques. In the Statement of Work, we had proposed to score ligand-regulated, intracellular co-localization of ER-RFP and GFP-co-factor fusions by visual inspection, as we had done in preliminary published studies (14). Instead, we developed more objective and far more precise co-

localization measurements that used computer-assisted quantification of red and green fluorescence intensities at each pixel within a digital image.

Co-localization is now accurately and reliably expressed as a mathematical function of the degree to which red and green fluorescence correlates on a pixel-by-pixel basis. With accurate quantification of fluorescence, we also were able to measure extent of interaction (and interaction kinetics) between the fluorophore-tagged molecules as the extent to which energy was transferred from one fluorophore to another, instead of being emitted as light (15). This procedure was perfected under this funding and was added to all of the data collection and analytical protocols being conducted to achieve the goals of our Statement of Work. We are now measuring fluorescence co-localization and the kinetics of interactions at individual pixels within a living cell by a simple one second data capture procedure, followed by a ten-second, semi-automated, computer analysis. With these improved procedures, we were able to measure intriguing, subtle differences in the actions of different SERMs provided to us by the collaborating laboratory of Dr. Tom Scanlan at U.C.S.F.. These advances are described in detail in the following section, together with the findings of our first year of study, which are soon to be submitted for publication.

Some initial difficulties were encountered for some of the first year project goals within our Statement of Work. These problems were associated with the development of new technologies and required some effort to resolve. This additional work included findings that:

1) the co-expression of RFP-labeled ER with some GFP-labeled co-factors was not well tolerated by the breast cancer cells we initially set out to study. We now realize that this is a factor that operates in a cell-specific fashion. It took extensive time to distinguish this effect from routine experimental difficulties. In fact, for some GFP-labeled peptides co-expressed with ER α -RFP, there was a breast-cancer cell-specific failure to grow or survive. This may be of significant therapeutic value. We have commenced a collaboration with the laboratory of Dr. Kip Guy (U.C.S.F.), which is developing small molecule analogs of these peptides.

2) Our initial attempts to follow the time course of interaction also were confounded by the difficulties of tracking a two dimensional focus within a cell that changes shape in three dimensions. This was addressed by purchasing an automated stage for our imaging system that allows us to follow cells in three dimensions, with time. This stage also will be used together with "cellular array" procedures we are currently developing. Our goal is to simultaneously measure, at each time point, the co-localization and interactions of a variety of combinations of RFP-ER and GFP-linked co-factors dotted onto adjacent spots in a slide. The microscope stage will move rapidly to each spot such that time-course data will be collected for multiple different combinations within a single experiment. This system is currently being set up and will be ready to speed our analysis in the second year of the project.

Findings: SERM-selective Dimerization of Estrogen Receptors

For characterizing the actions of SERMs on ER interactions and function, we constructed a string of computer commands that allowed us to:

- 1) quantify the extent of co-localization between different fluorophores as correlation co-efficients based upon background-subtracted fluorescence measurements from individual pixels;
- 2) quantify the amount of fluorescence resonance energy transfer (FRET) from a donor fluorophore to an acceptor fluorophore, again at individual pixels within each image;
- 3) quantify the amount of energy transfer, normalized to the amounts of acceptor and donor present at each pixel; this normalized FRET provides information about
 - a) the interaction kinetics of ER and its interacting partners
 - b) the distance separating the fluorophores
 - c) the rotational constraints imparted to the fluorophore by the attached proteins;
- 4) quantify the amounts of fluorescence at marked structures within the cell and compare the co-localization and direct interactions (measured by FRET) at different structures.

This analytical package was used in initial studies of the effects of different SERMs on the interactions of ER α with itself and with ER β . For the co-expression of ER α -RFP with ER α -GFP or ER α -RFP with ER β -GFP, correlation coefficients were very high (see Table I) and were not statistically different in the absence or presence of any of the ligands examined. The stable correlation coefficients indicated that the RFP- and GFP-labeled proteins were targeting to the same subcellular location regardless of the ligand present. This was to be expected for ER α -RFP and ER α -GFP since RFP and GFP were attached to the same protein, ER. Thus, high correlation coefficients for ER α -RFP and ER α -GFP co-localization validated the procedures for quantifying co-localization. The similar correlation co-efficients for ER α -RFP and ER β -GFP indicated that ER β -GFP localized to the same subnuclear location as did ER α -RFP regardless of ligand, which had not been previously known.

TABLE I. Regulation of Intranuclear Co-localization and Interaction by Existing SERMs (correlation coefficient of co-localization: mean +/- standard deviation) (interaction efficiency measured by FRET: 95% confidence intervals)				
	ERα-RFP/	ERα-GFP		ERα-RFP/
	correlation	interaction		ERβ-GFP
	coefficient	efficiency		interaction
No Ligand	0.87 +/- 0.06	7 to 10		coefficient
Estradiol	0.91 +/- 0.04	17 to 19		efficiency
Tamoxifen	0.89 +/- 0.05	15 to 17		8 to 9
4-hydroxy tamoxifen	0.90 +/- 0.05	16 to 19		0.88 +/- 0.05
Raloxifene	0.88 +/- 0.06	16 to 19		15 to 17
ICI 182,780	0.88 +/- 0.06	20 to 23		0.89 +/- 0.05
Genistein	0.89 +/- 0.05	12 to 14		16 to 18
				16 to 18

To determine the extent of interaction between the RFP- and GFP-labeled ERs, the amount of energy transferred from GFP to RFP on excitation of GFP was calculated, then normalized for the amount of GFP and RFP present. These procedures are described in a recent publication (15) from our laboratory that was supported by a DAMD Concept grant. There was some interaction (i.e. dimerization) of ER α -RFP with ER α -GFP and of ER α -RFP with ER β -GFP even in the absence of any ligand. This dimerization was indicated by an “interaction efficiency” significantly greater than zero. Each of the SERMs listed in Table I enhanced dimerization of ER α -RFP with ER α -GFP and of ER α -RFP with ER β -GFP. Notice that the soy isoflavone genistein was significantly poorer at promoting dimerization of ER α -RFP with ER α -GFP than were any of the other ligands. Genistein was, however, just as effective as the remaining SERMs at promoting dimerization of ER α -RFP with ER β -GFP. Conversely, ICI 182,780 was more effective than the remaining SERMs at promoting ER α homo-dimerization.

The ability to distinguish such subtleties in the interactions between factors, directly within the physiologic environment, can be very beneficial for dissecting the molecular and pharmacologic basis of SERM-specific actions. We next probed the effects of different chemical modifications to tamoxifen and genistein on the ability of these compounds to regulate dimerization (Table II). All derivatives of tamoxifen behaved similarly to tamoxifen (left side of Table II). Intriguingly, the strategic addition of a phenyl group caused one genistein derivative to be transformed into a ligand that regulated dimerization with properties similar to estradiol (Table II, compare interaction efficiencies for each ligand on the right side of the Table).

TABLE II. Effect of Novel SERMs on ERα-RFP/ERα-GFP Dimerization (interaction efficiency measured by FRET: 95% confidence intervals)			
	interaction efficiency		interaction efficiency
Tamoxifen	15 to 17	Genistein	12 to 14
GW5638	14 to 18	6,4'- dihydroxyflavone	11 to 13
GW5638-NH₂	15 to 19	phenyl- dihydroxyflavone	16 to 19
GW7604	15 to 18		
GW7604-keto	17 to 20	Estradiol	17 to 19

The automated data analysis that we have developed indicated not only the average amount of interaction between GFP and RFP-labeled factors (Table II). It also measured the specific amounts of interaction in each of thousands of individual pixels present within the cell nucleus. This enabled us to determine whether the effects of each SERM were uniform throughout the cell nucleus, or whether the effects of each SERM were limited to specific subnuclear structures. To measure of the intranuclear variation in ER α /ER α (Fig. 1) and ER α /ER β (not shown) dimerization, we determined the interaction efficiency at each pixel within more than one hundred cell nuclei for each treatment, then plotted the proportion of pixels containing each interaction efficiency. Note that we accurately measured here the interactions between approximately on five estrogen receptor dimers, on average, within each pixel.

In the absence of ligand, a significant number of pixels showed no evidence of interaction even though they contained both GFP and RFP-labeled ERs (Fig. 1, open circles). The addition of estradiol (black boxes) increased the proportion of pixels in which dimers were observed and resulted in stronger interaction within each pixel. This is characteristic for each ligand: as in the total cell interaction measurements (Table I), ICI 162,780 was more efficient (open triangles), and genistein (X's) was less efficient, than estradiol at both components.

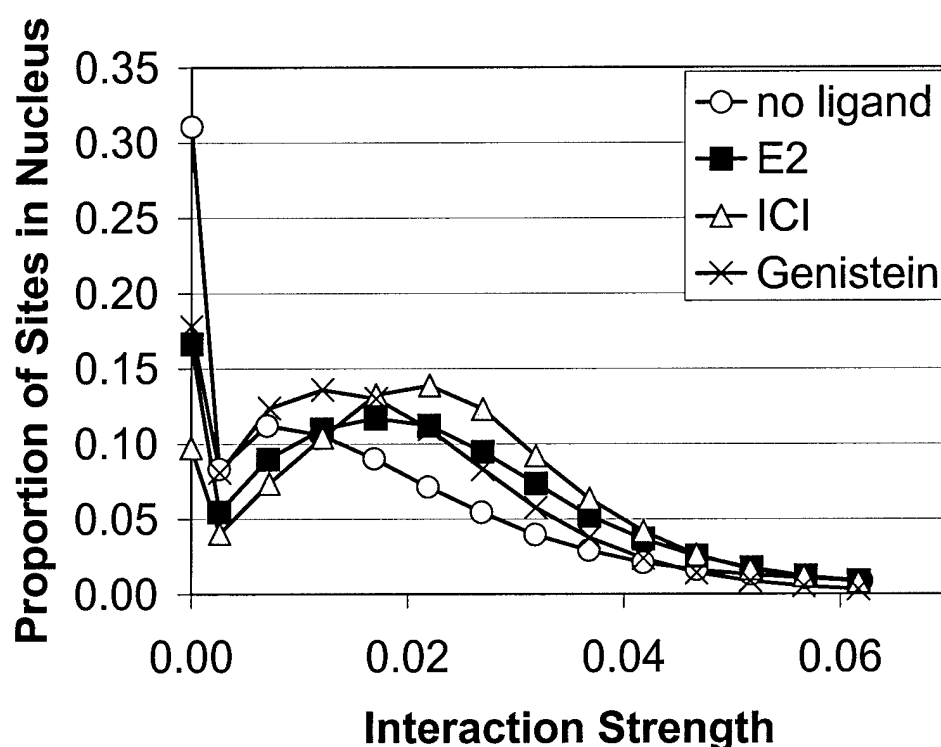


Fig. 1. Different SERMs affect both the strength of ER α dimerization at individual sites within the nucleus, as well as the number of sites showing any interaction. Interaction strength was measured by normalizing the amount of FRET for the amounts of GFP and RFP-labeled ER α present at each pixel within each nucleus.

Although tamoxifen and 4-hydroxy tamoxifen were not statistically different in their effects on the average interaction measured throughout a nucleus (Table I), these two ligands were considerably different when analyzed at individual pixels (Fig. 2). Tamoxifen showed a greater tendency to increase the number of pixels displaying dimerization and a poorer tendency to alter the extent of dimerization in the remaining pixels than did 4-hydroxy tamoxifen. Thus, pending our ongoing development of appropriate statistical techniques to correctly analyze this difference, tamoxifen and 4-hydroxy tamoxifen regulated dimerization differently at localized sites within the cell.

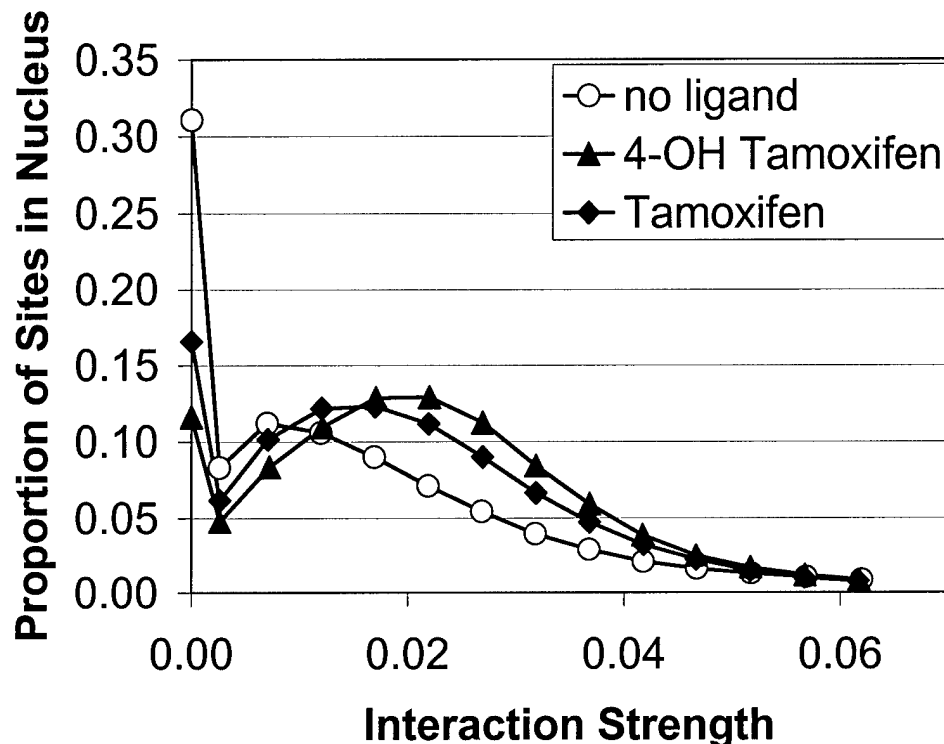


Fig. 2. Despite similar effects on overall interaction strength within a nucleus, tamoxifen and 4-hydroxy tamoxifen display unique patterns when their effects on ER α dimerization are determined at individual sites within the nucleus.

We (14, 15) and (16, 17) others previously reported that ER α concentrates at distinct intranuclear locations when bound to ligand. We therefore incorporated protocols within our data analysis package that selected pixels containing concentrated ER. We then measured the interaction strength at those pixels. The amount of FRET, normalized for the amount of RFP-ER α and GFP-ER α present, was not statistically different at the intranuclear structure where ER α concentrated from the amount of normalized FRET measured in the remainder of the nucleoplasm. This was true for every ligand examined and for both ER α /ER α and ER α /ER β dimers. Thus, there was no difference in ER dimerization strength at and away from concentrated ER that correlated with different intranuclear distributions of the tamoxifen and 4-hydroxy tamoxifen-bound ER α dimers.

Thus, we have developed a set of highly advanced imaging protocols and image analysis procedures that measure unprecedented details of the direct interactions and co-localization of ERs within living cells. These protocols are now ready to be used, in combination with medium throughput FRET array techniques we are currently developing, to rapidly compare a variety of SERMs for their effects on a broad spectrum of ER/co-factor interactions.

KEY RESEARCH ACCOMPLISHMENTS

The accomplishments listed below are complete and are currently being written for publication:

- 1) Estradiol, tamoxifen, 4-hydroxy tamoxifen and raloxifene all promote dimerization of ER α in living cells.
- 2) ER α dimerization is promoted better by ICI 182,780 than by the other compounds listed in Tables I and II.
- 3) ER α dimerization is promoted more poorly by the soy isoflavone genistein than by the other compounds.
- 4) All of the compounds listed in Table I promote the same amount of hetero-dimerization between ER α and ER β , despite their different effects on ER α homo-dimerization.
- 5) Four novel, chemical relatives of tamoxifen behave exactly as tamoxifen and 4-hydroxy tamoxifen in promoting average ER α homo-dimerization throughout the cell.
- 6) Tamoxifen and 4-hydroxy tamoxifen display subtle differences in the intranuclear distributions of the ER dimers they promote.
- 7) One chemical derivative of genistein behaves exactly as genistein in being a poor activator of ER α homo-dimerization.
- 8) Another modification of genistein (containing one additional attached phenyl group) is converted to promote ER α dimerization as effectively as estradiol, tamoxifen, 4-hydroxy tamoxifen, the tamoxifen derivatives or raloxifene. This acts as a chemical marker for the types of functional groups and interactions that affect how a compound regulates isoform-selective dimerization in living cells.

REPORTABLE OUTCOMES

One manuscript, derived from our work in the first year of this project is already being written for publication.

These techniques have been utilized in studies leading to at least one additional manuscript reporting the ligand-regulated interactions other nuclear receptors (the class of transcription factor to which the estrogen receptor belongs). The data for these studies have already been collected and statistically analyzed to completion. These manuscripts are currently in preparation and will acknowledge DAMD17-01-1-0190 support.

Research supported from DAMD17-01-1-0190 support, has also been reported in a number of talks by the Principal Investigator (Appendices 1-8) including:

- An invited lecture at the University of Michigan, Ann Arbor, MI, December 12, 2001;
- An invited lecture at the California Breast Cancer Research Program annual meeting, Oakland, CA, March 9, 2002;
- An invited lecture at the American Association for Cancer Research annual meeting, San Francisco, CA, April 8, 2002;
- An invited lecture at the Keystone Symposium on Nuclear Receptor Function, Snowbird, UT, April 14, 2002;

-An invited lecture at the U.C.S.F. Center for Reproductive Sciences annual retreat, Tiburon, CA, April 29, 2002;

The Principal Investigator will also include this data in invited talks scheduled for June at the Susan G. Komen Breast Cancer Foundation Meeting (Washington, DC) and at the annual meeting of the Endocrine Society (San Francisco, CA)

The FRET techniques developed with this proposal forms the basis of a number of different projects in a number of grant applications. This includes:

- a new DAMD fellowship application for Dr. Xin Lu that follows up novel aspects of her ER dimerization research not previously included in the Statement of Work,
- two new applications to the National Institutes of Health on parallel studies of other nuclear receptors (the class of protein to which ER belongs),
- a renewal application to the National Institutes of Health for work on the molecular interactions involved in pituitary cell differentiation,
- a shared instrumentation grant application to the National Institutes of Health for the purchase of second generation equipment to measure co-localization and FRET in three dimensions and with time, and
- a UCSF intramural application to study the molecular interactions of factors involved in adipogenesis.

PERSONNEL RECEIVING PAY FROM DAMD17-1-01-0190

Xin Lu, M.D., Ph.D.:	Post-Doctoral Scientist
Fred Schaufele, Ph.D.:	Principal Investigator
Vanya Shah, Ph.D.:	Post-Doctoral Scientist

(Dr Shah just joined the laboratory on April 1, 2002)

REPORTS AND PUBLICATIONS FROM DAMD17-1-01-0498 FUNDING

All meeting reports and publications are included in Abstracts 1 to 8. Publications are currently being written from the results of experiments supported by DAMD17-1-01-0190 funding.

CONCLUSIONS

We are now able to directly measure biochemical interactions in living cells. The significance of this is that we may now compare similar interactions under different cellular conditions to gain an understanding of the mechanisms by which SERMs regulated ER action cell-specifically. The power of the technique was highlighted by our studies in which we distinguished minor differences in the ability of SERMs to promote dimerization. Particularly significant was the ability to detect a novel pharmacophore that rescued reduced dimerization promoted by genistein. Furthermore, the technique itself is amenable to virtually any study of interactions between molecules. As such, it is sure to find wide general use for the elucidation of virtually any biomolecular pathway involved in any disease state.

Although we have successfully proven the power of the technique, there are still aspects of the Statement of Work that we will pursue in the coming year. We will follow the interactions with time after ligand addition to determine whether the ligands differentially affect the temporal kinetics of interactions. Initial experiments were attempted but failed due to the difficulties of keeping a three-dimensional cell in two-dimensional focus during the automated collection period. This is being overcome by imaging automatically in multiple focal planes then selecting those planes in focus for further analysis. We are also investigating procedures for using our automated stage to more simultaneously collect time courses from multiple, different ER/co-factor combinations. Finally, outside of the scope of this project, techniques are now becoming available in which cells marked with fluorophores can be studied *in situ* in living mice. We have already contacted laboratories with such equipment and look forward to applying our FRET techniques on whole animals to study organ-selective interactions between ER and its interacting factors.

REFERENCES

1. Zumoff B 1998 Does postmenopausal estrogen administration increase the risk of breast cancer? Contributions of animal, biochemical, and clinical investigative studies to a resolution of the controversy. *Proc Soc Exp Biol Med* 217:30-7
2. Colditz GA 1998 Relationship between estrogen levels, use of hormone replacement therapy, and breast cancer. *J Natl Cancer Inst* 90:814-23
3. Jordan VC, Morrow M 1999 Tamoxifen, raloxifene, and the prevention of breast cancer. *Endocr Rev* 20:253-78
4. Osborne CK 1998 Tamoxifen in the treatment of breast cancer. *N Engl J Med* 339:1609-18
5. Assikis VJ, Jordan VC 1997 Risks and benefits of tamoxifen therapy. *Oncology* 11:21-3
6. Baldereschi M, Di Carlo A, Lepore V, Bracco L, Maggi S, Grigoletto F, Scarlato G, Amaducci L 1998 Estrogen-replacement therapy and Alzheimer's disease in the Italian Longitudinal Study on Aging. *Neurology* 50:996-1002
7. Barrett-Connor E, Wenger NK, Grady D, Mosca L, Collins P, Kornitzer M, Cox DA, Moscarelli E, Anderson PW 1998 Hormone and nonhormone therapy for the maintenance of postmenopausal health: the need for randomized controlled trials of estrogen and raloxifene. *J Womens Health* 7:839-47
8. Cauley JA, Seeley DG, Browner WS, Ensrud K, Kuller LH, Lipschutz RC, Hulley SB 1997 Estrogen replacement therapy and mortality among older women. The study of osteoporotic fractures. *Arch Intern Med* 157:2181-7
9. Hulley S, Grady D, Bush T, Furberg C, Herrington D, Riggs B, Vittinghoff E 1998 Randomized trial of estrogen plus progestin for secondary prevention of coronary heart disease in postmenopausal women. Heart and Estrogen/progestin Replacement Study (HERS) Research Group [see comments]. *JAMA* 280:605-13
10. Palacios S 1999 Osteoporosis: prevention with estrogens in women over 60. *Maturitas* 33:1-6

11. Pritchard KI 1998 Estrogen/hormone replacement therapy and the etiology of breast cancer. *Recent Results Cancer Res* 152:22-31
12. Waring SC, Rocca WA, Petersen RC, O'Brien PC, Tangalos EG, Kokmen E 1999 Postmenopausal estrogen replacement therapy and risk of AD: a population-based study. *Neurology* 52:965-70
13. Cummings SR, Eckert S, Krueger KA, Grady D, Powles TJ, Cauley JA, Norton L, Nickelsen T, Bjarnason NH, Morrow M, Lippman ME, Black D, Glusman JE, Costa A, Jordan VC 1999 The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. *Multiple Outcomes of Raloxifene Evaluation. JAMA* 281:2189-97
14. Schaufele F, Chang C-Y, Liu W, Baxter JD, Wan Y, Nordeen S, Day RN, McDonnell DP 2000 Temporally distinct and ligand-specific recruitment of nuclear receptor interacting peptides and co-factors to subnuclear domains containing the estrogen receptor. *Mol Endocrinol* 14:2024-2039
15. Weatherman RV, Chang C-Y, Clegg NJ, Carroll DC, Day RN, Baxter JD, McDonnell DP, Scanlan TS, Schaufele F 2002 Ligand-selective interactions of estrogen receptor detected in living cells by Fluorescence Resonance Energy Transfer. *Mol Endocrinol* 16:487-496
16. Htun H, Holth LT, Walker D, Davie JR, Hager GL 1999 Direct visualization of the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. *Mol Biol Cell* 10:471-86
17. Stenoien DL, Mancini MG, Patel K, Allegretto EA, Smith CL, Mancini MA 2000 Subnuclear trafficking of estrogen receptor- α and steroid receptor coactivator-1. *Mol Endocrinol* 14:518-534

APPENDICES

- 1-8 . Abstracts for meetings in which supported data was, or is to be, discussed.

APPENDIX 1

Abstract for California Breast Cancer Research Program Meeting. Poster and talk presented March 9, 2002 in Oakland, CA

Novel Technologies to Identify Tissue-Selective Estrogens

Fred Schaufele (poster presenter), Xin Lu, Catherine Price, University of California, San Francisco

Breast tumors are broadly classified into two categories: those that contain, or do not contain, proteins that bind estrogens. Breast tumors that contain these proteins, called “estrogen receptors”, grow in the presence of an estrogen. The estrogen receptor does not function by itself but rather works by interacting with other proteins called “co-factors”. The types of co-factors that interact with the estrogen receptor change when estrogen binds the estrogen receptor. Thus, the estrogen-regulated interaction of co-factors with the estrogen receptor determines whether the tumor cell grows or not.

Certain drugs, including tamoxifen, block the growth of estrogen receptor-containing tumors. These drugs bind to the estrogen receptor and cause it to behave like the estrogen receptor without estrogen. However, some co-factors that interact with the estrogen receptor bound by tamoxifen also interact with the estrogen receptor bound by estrogen. Thus, tamoxifen blocks some co-factor interactions, particularly those required for the growth of breast tumors, but allows other co-factor interactions that cause the growth of some other tissues. In patients treated with tamoxifen, there is an increased risk of tumors in those other tissues, particularly the uterus.

Defining these co-factor interactions is the key to developing improved drugs not containing side effects. We developed novel cellular imaging technologies to follow the estrogen- and tamoxifen-induced changes in estrogen receptor interactions with itself and with specific co-factors in living cells. Briefly, we introduce into cells estrogen receptor and interacting proteins marked with different dyes that are activated by light of specific wavelengths to emit light of longer wavelengths. The activation and emission wavelengths of the different dyes partially overlap such that they cross-talk with each other. The degree of cross-talk tells us whether the tagged molecules interact and, if they do, how they interact and what shape the molecule attains.

Our long-term goal is to associate specific estrogen receptor/co-factor interactions with the desirable and undesirable clinical effects of each drug, then use this detailed knowledge to identify novel drugs that affect different subsets of those interactions. We present here our work, to date, characterizing these estrogen receptors interactions promoted or blocked by a number of clinically proven drugs and novel compounds. Ultimately, compounds with highly selective co-factor specificities may be identified that improve breast cancer treatment and/or minimize the side effects of current therapies. An ideal SERM could even be administered for decades to prevent breast cancer.

APPENDIX 2

Abstract for American Association for Cancer Research Annual Meeting. Talk presented April 8, 2002 in San Francisco, CA

Cellular imaging to identify ligands that modulate selected estrogen receptor actions

Fred Schaufele, Catherine Price, Xin Lu, University of California, San Francisco, San Francisco, CA.

Breast tumors are broadly classified into two categories: those that contain, or do not contain estrogen receptors (ER). In ER-containing tumors, growth occurs when estrogens bind to, and change the conformation of, ER. Tamoxifen, which is effective in slowing the growth of estrogen-dependent tumors, also binds ER, but causes it to change to a different conformation. These different ER conformations result in different co-factor interactions with the tamoxifen- or estrogen-bound ER such that tamoxifen acts as an anti-estrogen in some tissues but as an estrogen in others. We developed novel imaging technologies to follow the estrogen- and tamoxifen-induced changes in ER interactions with specific co-factors in living cells. We labeled the two known ERs and each co-factor with fluorescent tags of different colors and transferred them into cells. Fluorescence from each receptor and co-factor was quantified within 100 x 100 nm sections of the cell. Following treatment with estradiol, tamoxifen or 15 other "selective estrogen receptor modulators" (SERMs), we determined if the co-factor became more or less concentrated at the location of the estrogen receptor and if the proteins were so close that energy transferred between the attached fluorophores. We identified a number of different effects of different ER ligands on ER conformation and interaction. Each ligand modulated selected ER activities, but our measurement of these activities within living cells further distinguished spatial and temporal components of each ligand's action. Some ligands modulated ER conformation or co-factor interactions within localized regions of the nucleus. Other ligands modulated the number of sites within the nucleus at which specific conformations or interactions were detected. Our long-term goal is to associate specific ER conformation and co-factor interactions with the desirable and undesirable clinical effects of each drug. This detailed knowledge will be used to identify novel drugs that affect different subsets of those interactions. Some of these highly selective compounds may improve breast cancer treatment and/or minimize the side effects of current SERM therapies. An ideal SERM could even be administered for decades to successfully prevent breast cancer and to provide risk-free estrogen replacement therapy for post-menopausal women.

APPENDIX 3

Abstract for Keystone Symposium, Nuclear Receptor Meeting. Talk presented April 14, 2002 in Snowbird, UT.

Cellular imaging of nuclear receptor action

F. Schaufele, X. Lu, C. Price, R. Calmon

University of California, San Francisco, San Francisco, CA

We developed novel imaging technologies to follow ligand-regulated changes in nuclear receptor (NR) conformation, dimerization or interactions with specific co-factors in living cells. Estrogen receptors (ER) and multiple NR-interacting co-factors were labeled with different fluorescent tags and expressed in cells. Fluorescence from each receptor and co-factor was quantified within 100 x 100 nm sections of the cell in the presence or absence of different ligands to determine which ligand caused which co-factor to concentrate at the location of the ER. Direct interaction of ER and the interacting co-factor was measured by fluorescence resonance energy transfer (FRET). The effect of ligand on ER conformation and dimerization similarly was measured by studying FRET between the ERs themselves. Some ligands altered the number of sites within the nucleus at which FRET was observed. Other ligands changed the efficiency of FRET detected at each site, indicating that the conformation of the dimer or of ER/co-factor interaction was changed. In contrast, TR homodimers were limited to a small number of subnuclear regions and were disrupted by thyroid hormone. Instead, TR preferred to form heterodimers with RXR in a ligand-independent fashion. Thus, the spatial and temporal characteristics of ligand regulation of NR conformation, dimerization and co-factor interaction can be determined in the physiologic environment of the living cell. This is correlated with the known transcriptional and clinical effects of each NR ligand to identify, with unprecedented detail, the molecular and cellular events underlying nuclear receptor action.

APPENDIX 4

Abstract for the U.C.S.F. Center for Reproductive Sciences Annual Retreat. Talk to be presented April 29, 2002 in Tiburon, CA.

Estrogen Receptor Dimerization and Interactions in Living Cells

Fred Schaufele, Xin Lu, Catherine Price

Estrogens regulate a number of tissues including those involved in reproductive functions. Women who have undergone menopause, or other estrogen-deficient patients, experience a decline in overall health related to the absence of tissue-selective regulation of the nuclear estrogen receptor (ER). Hormone replacement therapies are possible. However, complications arising from estrogen replacement include an increased risk of breast and endometrial cancers as well as venous thrombosis. As a result, long-term estrogen replacement therapies have not become widespread despite their potential for significantly improving overall health and quality of life.

An ideal long-term estrogen replacement therapy would block ER actions in specific tissues while promoting ER action in other tissues. This requires methods for detecting the fine details of ER action in different cell types in response to each selective ER modulator (SERM). We developed novel fluorescence microscopy techniques for studying ER interaction within living cells. ER and ER-interacting factors are tagged with red and green fluorophores and their relative intracellular positions are determined upon incubation with SERMs. The extent to which energy is transferred from the green fluorophore to the red fluorophore identifies direct interactions between ER and each co-factor at specific intracellular locations within each cell type examined.

Using our fluorescence co-localization and resonance energy transfer (FRET) techniques, we determine, for example, that some ligands, including estradiol, tamoxifen and the soy isoflavone genistein, all increase the number of intranuclear sites at which ER α homodimers and ER α /ER β heterodimers are found. FRET measurements also show that the estradiol and tamoxifen-bound dimers are kinetically, or conformationally, different from the genistein-bound dimers. In contrast, estradiol, tamoxifen and genistein each promote interactions and co-localization with different subsets of co-factors. Our goal is to establish a "fingerprint" of the conformational and interactive consequences of each ligand to the ER in different ER-responsive tissues. These fingerprints will be compared to the known clinical effects of each compound. This will aid the identification of ER ligands with unique tissue-selective activities, some of which may prove useful for safe, long-term, post-menopausal hormone replacement therapies. Some of these ligands may even find a role in pre-menopausal breast and uterine cancer prevention.

APPENDIX 5

Abstract for the Susan G. Komen Breast Cancer Foundation Meeting. Talk to be presented June 3, 2002 in Washington, DC.

Cellular Imaging to Identify SERMs Improved for Breast Cancer Therapy

Fred Schaufele, Xin Lu, Catherine Price

Breast tumors are broadly classified into two categories: those that contain, or do not contain, proteins that bind estrogens. Breast tumors that contain these proteins, called “estrogen receptors”, grow in the presence of an estrogen. The estrogen receptor does not function by itself but rather works by interacting with other proteins called “co-factors”. The types of co-factors that interact with the estrogen receptor change when estrogen binds the estrogen receptor. Thus, the estrogen-regulated interaction of co-factors with the estrogen receptor determines whether the tumor cell grows or not.

Certain drugs, including tamoxifen, block the growth of estrogen receptor-containing tumors. These drugs bind to the estrogen receptor and cause it to behave like the estrogen receptor without estrogen. However, some co-factors that interact with the estrogen receptor bound by tamoxifen also interact with the estrogen receptor bound by estrogen. Thus, tamoxifen blocks some co-factor interactions, particularly those required for the growth of breast tumors, but allows other co-factor interactions that cause the growth of some other tissues. As a result, in patients treated with tamoxifen, there is an increased risk of tumors in those other tissues, particularly the uterus.

Defining these co-factor interactions is the key to developing improved drugs not containing side effects. We developed novel cellular imaging technologies to follow the estrogen- and tamoxifen-induced changes in estrogen receptor interactions with specific co-factors in living cells. Our long-term goal is to associate specific estrogen receptor/co-factor interactions with the desirable and undesirable clinical effects of each drug, then use this detailed knowledge to identify novel drugs that affect different subsets of those interactions. Ultimately, compounds with highly selective co-factor specificities may be identified that improve breast cancer treatment and/or minimize the side effects of current therapies. An ideal SERM could even be administered for decades to prevent breast cancer.

APPENDIX 6

Abstract for the Endocrine Society Annual Meeting. Talk to be presented June 22, 2002 in San Francisco, CA.

Conformation and Interactions of Transcription Co-Regulatory Factors at Discrete Subnuclear Loci Revealed by FRET Nanoscopy

Fred Schaufele, Xin Lu, Raphael Calmon

University of California, San Francisco, CA, 94143-0540

John F. Enwright III

Austin College, Sherman, TX, 75090

Richard N. Day

University of Virginia, Charlottesville, VA, 22908-0578

Transcription factors direct co-regulatory complexes to genes buried within the genome. Compartmentalization of gene regulatory factors in the highly organized nucleus may affect their structure at and/or access to specific genes. By fluorescence microscopy, we determined the subnuclear locations of a number of transcription factors and co-regulatory factors that control gene expression and differentiation in multiple cell types. Expression of the transcription factor C/EBP[alpha], which is absent from pituitary and adipocyte progenitor cells, was accompanied by a highly selective recruitment of the co-activator CBP and acetylated histone H3 to the peri-centromeric chromatin, where C/EBP[alpha] concentrated. In the pituitary model, the expression of a second transcription factor, Pit-1, dispersed C/EBP[alpha] away from the peri-centromeric chromatin. Nuclear receptors also altered the location of specific co-regulatory factors, in a ligand-regulated fashion.

To study the corresponding biochemical events associated with intranuclear reorganization, we developed fluorescence resonance energy transfer (FRET) techniques that precisely measure the conformations and interactions of co-localized molecules at each of thousands of sites within living cells. C/EBP[alpha] formed dimers effectively at most locations throughout the nucleus. Tagging different domains of C/EBP[alpha], and measuring the amount of inter-domain FRET, demonstrated that the conformation of C/EBP[alpha] was different at the peri-centromeric chromatin than in the rest of the nucleus. Treating the cells with an activator of protein kinase C changed the structure of C/EBP[alpha]. For some nuclear receptors, we found that dimers were distributed unevenly throughout the nucleus. Cognate ligands increased (estrogen receptor [alpha] and [beta] homo and heterodimers), decreased (thyroid hormone receptor homodimer) or had little effect on (RXR[alpha] homodimer, TR[beta]/RXR[alpha] heterodimer) the number of subnuclear sites at which interactions occurred. Some ligands also changed the extent of interaction at each site. For the ER, we found differences in the types of dimerizations and interactions promoted by different ligands used in breast cancer therapy. Thus, the interactions of conformations of gene regulatory complexes are determined by intranuclear location. The re-location of co-regulatory factors by some transcription factors may regulate the patterns of gene expression in differentiation.

APPENDIX 7

Abstract submitted for the CDMRP Breast Cancer Research Program Meeting, September 25-28, Orlando, FL.

Cellular Imaging Technologies for Identifying Tissue-Selective SERMS

Fred Schaufele, Catherine Price, Xin Lu

Tamoxifen is an estrogen receptor (ER)-binding compound effective for blocking the proliferation of many ER-containing breast tumors. With its anti-estrogenic effects, tamoxifen blocks also the beneficial effects of estrogens in some tissues. Tamoxifen also acts like an estrogen in some tissues in which anti-estrogenic activities would be preferred. These undesired estrogenic and anti-estrogenic side effects limit the use of tamoxifen for breast cancer prevention only to high-risk patients.

An ideal treatment for breast cancer would block ER actions in specific tissues while promoting ER action in other tissues. This requires methods for detecting the fine details of ER action in different cell types in response to each selective ER modulator (SERM). We developed novel fluorescence microscopy techniques for studying ER interaction within living cells. ER and ER-interacting factors are tagged with red and green fluorophores and their relative intracellular positions are determined upon incubation with SERMs. The extent to which energy is transferred from the green fluorophore to the red fluorophore identifies direct interactions between ER and each co-factor at specific intracellular locations within each cell type examined.

Using our fluorescence co-localization and resonance energy transfer (FRET) techniques, we determine, for example, that some ligands, including estradiol, tamoxifen and the soy isoflavone genistein, all increase the number of intranuclear sites at which ER α homodimers and ER α /ER β heterodimers are found. FRET measurements also show that the estradiol and tamoxifen-bound dimers are kinetically, or conformationally, different from the genestein-bound dimers. In contrast, estradiol, tamoxifen and genestein each promote interactions and co-localization with different subsets of co-factors. Our goal is to establish a "fingerprint" of the conformational and interactive consequences of each ligand to the ER in different ER-responsive tissues. These fingerprints will be compared to the known clinical effects of each compound. This will aid the identification of ER ligands with unique tissue-selective activities, some of which may prove useful for safe, long-term breast cancer prevention therapies.

APPENDIX 8

Abstract submitted for the CDMRP Breast Cancer Research Program Meeting, September 25-28, Orlando, FL.

Kinetics of Estrogen Receptor Action in Cell Types Relevant to Breast Cancer

Fred Schaufele, Xin Lu

The proliferation of a subset of breast tumors depends upon circulating estrogens. These tumors generally contain the estrogen receptor (ER), which is a target for the anti-breast cancer drug tamoxifen. However, tamoxifen is not an ideal drug, and its side effects in non-breast tissues limit its use in preventative therapies.

Our goal is to understand the tissue-selective actions of estrogen action. We have developed novel fluorescence imaging techniques that precisely define the molecular actions of ERα and ERβ in cultured tumor cells. These techniques apply a physical principle, in which energy is transferred from a fluorophore ("GFP") attached to ER to a second fluorophore ("RFP") attached to nearby ER. This has allowed us to precisely track ER dimerization in living cells. With these techniques, we have characterized the effects on ER dimerization of estradiol and certain selective estrogen receptor modulators (SERMs), including tamoxifen, raloxifene, ICI 182780, the soy isoflavone genestein, multiple derivatives of genestein and diethylstilbestrol.

At individual pixels within each image captured from a cell, we quantify the amount of ER-GFP, ER-RFP and energy transfer (FRET). We also calculate the amount of FRET normalized for the amounts of ER-GFP and ER-RFP present. This "efficiency" of FRET reflects the amount of contact between ER within the dimer and informs us of the kinetics of the interaction.

In the absence of ligand, there is some FRET indicating some dimerization. In the presence of any of the above ligands, the amount of FRET increases. In particular, for genestein and derivatives, the amount of FRET increases because there is an increase in the proportion of pixels (i.e. sites within the nucleus) at which ER dimerizes. This increase in the number of dimerization sites also was observed for the other ligands. For these ligands, there also was an increase in FRET efficiency at each site. Thus, these ligands also alter the dimer interaction itself. We also have applied FRET to identify SERM-selectivity in ER interactions with target proteins. By mapping fine distinctions between the molecular actions of each SERM, we aim to identify novel SERMs with improved specificity for breast cancer treatment and prevention.